

***IN THE UNITED STATES PATENT AND TRADEMARK OFFICE***

Applicant: Himanshu BRAHMBHATT *et al.*

Title: **PHARMACEUTICALLY COMPATIBLE METHOD  
FOR PURIFYING INTACT BACTERIAL MINICELLS**

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**DECLARATION UNDER 37 C.F.R. § 1.132**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

I, Himanshu Brahmbhatt, declare the following:

1. I am a co-inventor named in the captioned application.
2. I am a cofounder of EnGeneIC Molecular Delivery Pty Ltd and have served as a Joint-CEO and Director of the company since its inception in February 2001. I received my Ph.D. in 1987 at the University of Adelaide, South Australia. I subsequently carried out my post-doctoral research at the Dept. de Biochimie Medicale, Centre Medicale Universitaire, Geneva, Switzerland (June 1987 - June 1988) and the National Centre for Research in Biotechnology, Braunschweig, Germany (July 1988 - Aug. 1991). I then served as a Research Scientist (Sept. 1991 - June 30th, 1994), a Senior Research Scientist (July 1, 1994 – June 30<sup>th</sup>, 1999), and a principal Research Scientist (July 1, 1999 – January 15, 2001) at CSIRO McMaster Laboratory, Division of Animal Health (Sydney, Australia). I have spent much my career studying bacterial vaccines, parasite vaccines, and cancer biology. I have over 12

publications in those and related fields, with most of my research output reflected in various patent applications.

3. I understand that claims 9, 11-12, and 27-44 of the above-referenced case are rejected over Khatchatourians et al. in view of Christen et al. More specifically, I understand that the examiner seeks empirical data demonstrating that Khatchatourians' notion of preparing preparations of minicells in fact does not yield such a preparation.

### Aims

4. Khachatourians reported that minicells were resistant to sonication while bacterial cells were sensitive. Accordingly, he suggested that sonication could be used in a methodology for purifying minicells from parent bacterial contaminants. That is, Khachatourians suggested that, following sonication, the bacterial contaminants would lyse and be eliminated, leaving intact minicells in place.

5. My co-inventor and I conducted the experiments described below, in order to test Khachatourians' premise that "sonic treatment disrupts whole cells" and "does not affect minicells". In particular, we endeavored to determine the effect of various levels and duration of sonication treatment on a suspension of minicells and bacteria.

6. SYTO<sup>®</sup>9 is a nucleic acid fluorescent stain (bright green fluorescence) that is able to enter all cells through an intact cellular membrane. Intracellularly, it binds to nucleic acids such as DNA and RNA and this results in a bright green fluorescence of the stained intact cell. SYTO<sup>®</sup>9 is available from Invitrogen (Carlsbad, CA, USA) and routinely used by researchers to determine live versus dead bacterial cells or bacterial cells that have damaged membranes. See, e.g., Berney et al., *Applied and Environmental Microbiology* 73: 3283-90 (2007) (Exhibit 1), and LIVE/DEAD<sup>®</sup> BacLight<sup>TM</sup> Bacterial Viability and Counting Kit, catalogue number L34856, Invitrogen (Exhibit 2).

7. The aim of this experiment was to determine whether bacteria and minicells were damaged following mild sonication. If the membrane integrity were damaged and the bacterial cells or minicells were lysed, then the SYTO<sup>®</sup>9 dye would not be retained in the

cells due to formation of cellular debris. Consequently, the number of bacterial cells or minicells counted by FACS analyses would be expected to decrease after sonication, as bacterial cells or minicells lysed.

### **Methodology**

8. The minicells or bacteria were stained with SYTO<sup>®</sup>9 and counted on the Flow Cytometry (FACS). Cell debris should not be stained by SYTO<sup>®</sup>9, and so, if the minicells and bacteria are being disintegrated by sonication, then one expects the minicell count to decrease with longer durations of sonication.

9. The following procedures were followed:

- a)  $2 \times 10^8$  minicells or  $2 \times 10^9$  *Salmonella Typhimurium* bacteria in 1ml of 0.9% NaCl (physiological saline) were sonicated on ice at 10% output for the following durations:
  - 30 seconds
  - 1 minute
  - 2 minutes
  - 4 minutes
- b) The tubes were spun down at 16,100 x g and the supernatant removed. They were resuspended in 1ml 200 mM trehalose and vortexed thoroughly, then incubated at 37°C for 15 mins.
- c) 12.5µl of minicells, 2.5µl of Bacteria (0.5-2mins) and 5µl of Bacteria (4 mins) were added to a flow tube containing 482 µl Isoflow, 5 µl calibration beads, and 0.75µl SYTO<sup>®</sup>9 and incubated for 20 mins protected from light.
- d) The samples were run on the FACS using the FL1 channel to detect SYTO<sup>®</sup>9.

Minicells were counted via a Beckman FC500 Flow Cytometer and CXP software, using the minicell COUNT#3 program, with the following settings:

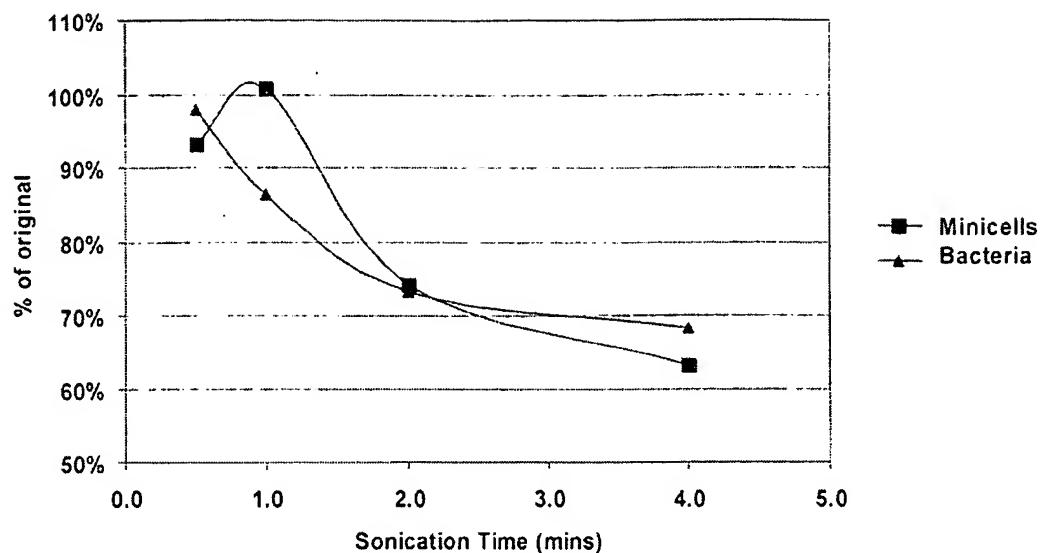
- Discriminator FL1 = 1.
- Count time = 60 seconds
- Flow rate: LOW (~10 µL/min)

	Volts	Gain
FS	440	7.5
SS	300	10.0
FL1	640	1.0

- The minicell region in the SS log vs FL1 log plot contains the minicells. The software calculates the minicell concentration in minicells per microliter ( $\mu\text{L}$ ). One then subtracts the saline blank from the minicell count, multiplies by the dilution factor and the beads:minicell volume ratio, and then multiplies 1,000 to give the minicell concentration per mL.

## Results

	LMD	CAL#	Minicells ( $\mu\text{L}$ )	Dilution	Beads:minicell	Minicells/ $\mu\text{L}$	Minicells/mL*
<b>Blank</b>	804	8331	1000	1.0		16,289	
<b>Minicells – sonication time</b>							
<b>30 seconds</b>	805	8116	1000	1.0	0.40	256,641	9.61E+08
<b>1 minute</b>	806	8268	1000	1.0	0.40	277,383	1.04E+08
<b>2 minutes</b>	807	9289	1000	1.0	0.40	204,123	7.51E+07
<b>4 minutes</b>	808	8577	1000	1.0	0.40	173,542	6.29E+07
<b>Bacteria – sonication time</b>							
<b>30 seconds</b>	817	10900	1000	1.0	2.00	636,651	1.24E+09
<b>1 minute</b>	814	9884	1000	1.0	2.00	561,159	1.09E+09
<b>2 minutes</b>	815	10238	1000	1.0	2.00	476,577	9.21E+08
<b>4 minutes</b>	816	10356	1000	1.0	1.00	443,482	8.54E+08



### Conclusion

10. The above results show that minicells exhibit significant sensitivity to sonication, *contra* Khachatourians. Post-sonication, the minicells and bacterial cells lose membrane integrity and lyse; this, despite use of the lowest sonication energy and duration of sonication (seconds to a few minutes).

11. By the same token, these results clearly show that Khachatourians' proposed method for minicell purification would result in seriously damaged minicells and a significant number of minicells would be lost through lysis. Khatchatourians employed higher sonication energies than what was tested here, for much longer periods of time, *i.e.*, up to 2 hours. Accordingly, it would have been apparent to the knowledgeable person in the field, *circa* 2003, that the Khatchatourians methodology was *not* usable for purifying bacterial minicells.

12. Under penalty of perjury under the laws of the United States of America, I declare that the foregoing is true and correct.

23<sup>rd</sup> DECEMBER 2008  
Date

  
Himanshu Brahmbhatt